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(54) Title: PLATELET STABILISATION

(57) Abstract: A method for the production of stabilised platelets, comprises the steps of: (i) pre-activating platelets, to induce the formation of microvesicles; (ii) contacting the pre-activated platelets with a carbohydrate, whereby the carbohydrate is incorporated into the platelets; and (iii) drying the thus-loaded platelets.

PLATELET STABILISATION

Field of the Inv ntion

This invention relates to methods for incorporating carbohydrates into platelets, to stabilise the platelets during storage.

Background to the Invention

Blood platelets are one of the complex components involved in maintaining haemostasis. When the vessel wall is damaged, platelets adhere to exposed surfaces composed of collagen, microfibrils, and basement membrane. Adherent platelets promote the recruitment of other platelets to form an aggregated mass called a haemostatic platelet plug. The result is to activate coagulation proteins which provide the network to stabilize the platelet plug and reduce bleeding, allowing tissue repairs to occur.

Platelets are transfused to patients for many clinical indications. For instance, platelet infusions are used to correct deficiencies or dysfunctions of circulating platelets as a result of trauma, disease, or drug induced dysfunction. Patients suffering from idiopathic thrombocytopenia and those undergoing ablative chemotherapy are treated with platelet infusions. The increasing use of ablative chemotherapy for a wide variety of malignancies has resulted in an increased need for replacement platelet therapy.

A major difficulty in using isolated platelets is their short shelf-life. Platelets are only approved by the Food and Drug Administration (FDA) for storage in a liquid state for up to five days at room temperature, during which time the functional properties rapidly deteriorate. This causes many logistic problems in both civilian and military medicine.

Further drawbacks of storing platelets in a liquid state include the necessity of considerable storage space and constant agitation within bags of specially developed gas permeable plastics. Typically, single buffy-coat-derived platelets are stored in a suspending plasma volume of 45 to 65 ml. Recently, a study reported liquid storage establishing a minimum plasma volume of 30-50 ml (Home *et al.* (1994) Transfusion 34:39; and Ali *et al.* (1994) Transfusion 34:44). This storage method still requires considerable space,

howev r, and the shelf life is not extended beyond approximately five days. Th major probl m with liquid storage is that the platelets need to be stored at approximately 20°C, as even short periods of exposure to lower temperatures during storage result in substantial changes in their *in vivo* and *in vitro* properties (Moroff G. *et al.* (1994) Transfusion 34:317). As this storage usually also requires agitation of the platelets during storage at about 20°C often in the presence of glucose, this presents optimal conditions for bacterial growth and this is a major problem with the storage of liquid platelets.

To minimise the problems of bacterial growth, refrigerated storage at 4°C or frozen storage at -80°C has been proposed. However, this requires methods to prevent the cold-activation of the platelets to be stored and the possible use of cryo-protectants. This method would however, require extensive washing of the platelets, to remove the cryo-protectants, before therapeutic use.

In addition, platelets are activated during the process of freeze drying and can only be used as a heamatology standard. The electrokinetic properties of the preserved platelets are different from those of fresh matched platelets. Other attempts at lyophilizing platelets have met with suboptimal results. Fixing the platelet prior to freeze-drying improves their function, but these freeze-dried platelets need to be stored frozen at -80°C. (Read *et al.* (1995) Proc. Natl. Acad. Sci. 92:397).

WO-A-98/34478 discloses methods for stabilising platelets by incorporating the carbohydrate trehalose into the platelets, and then drying in the presence of a stabilising carbohydrate. The preferred method for incorporating the trehalose into the platelets is electropermeabilisation. Although this technique does result in high uptake of trehalose, the technique has the disadvantage of activating the platelets, and it can only be applied on a small scale unless expensive equipment is used.

Summary of the Invention

The present invention is based on the realisation that platelets can be loaded with high concentrations of a carbohydrate sugar by pre-activating, but not fully activating, the platelets. Without wishing to be bound by theory, this

may involve inducing the platelets to form microv sicles which incorporate carbohydrates on their formation, and then allowing uptak of th microvesicl s by the platelets.

According to the present invention, a method for the production of stabilised platelets comprises the steps of:

(i) pre-activating platelets, to induce the formation of microvesicles;

(ii) contacting the pre-activated platelets with a carbohydrate, whereby the carbohydrate is incorporated into the platelets; and

(iii) drying the thus-loaded platelets.

The present invention provides a suitable method for high efficient loading of platelets with a suitable carbohydrate, which can be carried out on a large scale and is sufficiently gentle to prevent activation of the platelets. After incorporation, the platelets may be dried by any suitable means, preferably in the presence of an external carbohydrate, for storage and transport.

Description of the Invention

The present invention is based at least in part on the realisation that microvesicle formation can be used to incorporate carbohydrates into platelets, without activating the platelets. Microvesicles are formed on the pre-activation of platelets, which is a well defined mechanism that has apparently been developed for the communication of signal molecules such as CD62p. Pre-activation (a term which will be understood by those of ordinary skill in the art) can be induced by many methods, including treating the platelets to induce pressure or stress, e.g. shear stress by, for example, filtration. Leukocyte reduction filters are appropriate for use to induce pressure or stress, e.g. shear stress by filtration. Centrifugation and other changes in the environment of the platelets may also be used to induce pre-activation.

Platelets may be obtained by any method known in the art. Typically, platelets are obtained by collecting blood into a suitable anticoagulant followed by obtaining platelet-rich plasma (PRP) by any method known in the art. After

a platelet pell t is obtained by centrifugation, the pellet may be resuspended in a physiologically-acceptable solution, prior to pre-activation.

Typically, step (ii) of the method of the invention is carried out at a temperature of from 33°C to 41°C. Step (i), i.e. pre-activation, may also be conducted at such a temperature.

The novel method can be carried out so that there is no further activation of the platelets. This may typically be carried out by conducting steps (i) and (ii) at least in the absence of calcium or in the presence of a calcium-chelating agents, e.g. EGTA or EDTA. However, the addition of such agents is not preferred for clinical use, and is not necessary if the pH of the platelets does not drop too far, e.g. below 6, such that activation occurs.

Preferably, the carbohydrate to be incorporated is a non-reducing sugar, for example sucrose, trehalose or raffinose. Typically, the carbohydrate will be present in the composition at a concentration of from 5 mM-80mM, preferably 20mM-60mM and most preferably 40mM-50mM. More generally, the present method can be used to incorporate many different carbohydrates into platelets. The preferred carbohydrate used in the present invention is trehalose (α-D-glucopyranosyl-α-D-glucopyranoside).

After the carbohydrate has been incorporated into the platelets, the platelets may be resuspended in a drying buffer which typically contains a stabilising agent. The stabilising agent can be any carbohydrate that effects stabilisation of the dried platelets. Preferably the stabilising agent is trehalose. Drying the platelets may be carried out according to the description in WO-A-98/34478.

The following Example illustrates the invention.

<u>Example</u>

Platelet-rich plasma (PRP) or platelet concentrate (PC), having a platelet concentration of approximately 1-3 x 10^9/ml, was washed twice with a buffer (100mM NaCl, 10mM KCl, 10mM EGTA, 10mM imidazole, pH 7.4) with centrifugation at 480 g. The pellet was resuspended in the buffer which also

contains 30-50mM trehalose. Pre-activation was induced by filtering the plat l t concentrate, using a leukocyte reduction filter at 37°C.

After the filtering step, the platelets were incubated at 37°C for up to 5 hours (optimal time was 2-3 hours). During this time, microvesicles formed during pre-activation were loaded with trehalose.

Platelet counts were then obtained. The platelets were pelleted (45 seconds at 14000 rpm) and the sugars were extracted using 80% methanol. The methanol was then evaporated with nitrogen/air, and the sugar samples redissolved in H_2O, prior to analysis. The amount of trehalose in the platelets was quantified using the anthrone reaction (Umbreit *et al*, Manometric and Biochemical Techniques 5th Edition, 1972) and by HPLC.

As a control experiment, the enzyme trehalase was added and the glucose concentration was measured after washing the cells. The original amount of trehalose in the cells was calculated and appeared to be similar to the quantity measured by HPLC.

CLAIMS

1. A method for the production of stabilised platelets, comprising the steps of:

   (i) pre-activating platelets, to induce the formation of microvesicles;
   (ii) contacting the pre-activated platelets with a carbohydrate, whereby the carbohydrate is incorporated into the platelets; and
   (iii) drying the thus-loaded platelets.

2. A method according to claim 1, wherein step (i) comprises a physical change in the environment of the platelets.

3. A method according to claim 2, wherein step (i) comprises inducing shear stress on the platelets.

4. A method according to claim 3, wherein step (i) comprises filtering the platelets.

5. A method according to any preceding claim, wherein the carbohydrate is trehalose.

6. A method according to any preceding claim, wherein step (ii), and optionally also step (i), are carried out at a temperature of from 33°C to 41°C.

7. A method according to any preceding claim, wherein step (ii) is conducted in the presence of a platelet activation inhibitor.

8. A method according to claim 7, wherein the inhibitor is vitamin A.

9. A method according to any preceding claim, wherein steps (i) and (ii) are conducted simultaneously, i.e. the pre-activation is conducted in the presence of the carbohydrate.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 34478 A (GRANDAGE LYNDA MARY ;QUADRANT HOLDINGS CAMBRIDGE (GB); MENYS VALEN) 13 August 1998 (1998-08-13) cited in the application page 5, paragraph 3 -page 13, paragraph 2 ---	1-9
A	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; D.A.LEWIS: "Erythrocytes as microvesicles" retrieved from STN-INTERNATIONAL, accession no. 121:212705 CA XP002160827 abstract & MICROCAPSULES NANOPART. MED. PHARM., 1992, pages 299-313, --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 653 999 A (GAUDREAULT RENE C ET AL) 5 August 1997 (1997-08-05) column 1, line 66 -column 2, line 61	1-9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9834478	A	13-08-1998	AU	6220898	A	26-08-1998
			CN	1246778	T	08-03-2000
			EP	0967862	A	05-01-2000
			ZA	9801031	A	11-08-1998
US 5653999	A	05-08-1997	CA	2150617	A	08-08-1996